TITLE OF THE INVENTION

A NOVEL IMMUNO-DIAGNOSTIC TEST METHOD FOR VETERINARY DISEASE

FIELD OF THE INVENTION

The present invention is related to an apparatus and method for performing immunodiagnostic testing for veterinary disease. In particular it is related to the detection of the presence of viral or bacterial antigens by measuring specific antibodies in animal body fluid in which viral or bacterial proteins serve as the antigens. Highly specific antigen-antibody interactions are utilized to bind antibodies onto a piezoelectric crystal transducer for detection.

BACKGROUND OF THE INVENTION

The publications and other materials used herein to illuminate the background of the invention or provide additional details respecting the practice, are incorporated by reference, and for convenience are respectively grouped in the appended List of References.

In the veterinary diagnostic laboratory, the most common need for diagnostic testing is to diagnose disease, which usually involves testing for the presence of viral or bacterial antigens, or antibodies to them. Traditional methodologies practicing this art such as viral isolation, virus neutralization, plate agglutination, hemagglutination inhibition, immunodiffusion, classical microbiological culture techniques, high-pressure liquid chromatography, and thin layer chromatography, are very slow and costly, requiring days or even weeks for confirmation results. To accelerate and simplify current immunoassay techniques, the use of highly specific antigen-antibody reaction is becoming increasingly important. Techniques such as radioimmunoassay (RIA), immunofluorescence assay (IFA), enzyme-linked immunosorbent assay (ELISA), and Western immunoblot (WB) have shown considerable degrees of success and have are used in veterinary diagnostic laboratory nowadays.

Most of the said immunoassays are conducted in laboratories that use relatively expensive equipment and skilled personnel. Most tests involve manipulation and multiple procedures. For example, a traditional solid phase sandwich immunoassay for viral or bacterial antigen/antibody detection comprises the following procedures: (a) Immobilizing antibody/antigen peptide to a solid support, (b) Reacting immobilized antibody/antigen from step (a) with samples suspected of containing antigen/antibody against coated antibody/antigen to form an immunocomplex, (c) Washing the immunocomplex of step (b) with washing solution comprising detergents to remove

unbound antigen/antibody, (d) Reacting the immunocomplex with an enzyme or radiolabeled second antibodies to form a sandwich complex, (e) Separating the sandwich complex from unbound labeled second antibodies by washing, (f) Reacting the sandwich complex with a compound able to act as a substrate for the enzyme or radiolabel so that the catalytic reaction can be colorimetrically monitored.

The use of radioisotope- or enzyme-labeled second antibody in the assay procedures presents problems of waste handling and increases the cost of the assay. The multiple reaction processes and long incubation time require 8-10 hours from initial sample processing to delivery of final results. The requirements for refrigerated storage of reagents, expensive equipment, and reliable power supply make the assay techniques difficult to perform outside a well-equipped laboratory or under field conditions.

Attempts to overcome the limitations imposed by these requirements have led to the development of a variety of rapid immunoassay methods. For example, dot enzyme immunoassay (DEI), dot immuno-binding assay (DIA), and various particle agglutination tests are developed for the routine laboratory diagnosis for viral/bacterial serum antibodies. In these assays, antigens are dotted onto nitrocellulose strips or sheets, and sera are applied on absorbent paper strips. Antigen/antibody complexes are detected with enzyme-conjugated antiglobulin and development of a colored, insoluble substrate product. This test allows processing the serum detection within a relatively short time (1-5 h). Although these assays have improved the utility outside the laboratory environment, their usefulness is still somewhat limited by the potential temperature sensitivity inherent in enzyme-liked systems, and by the need for the specific anti-immunoglobulin reagents (Heberling and Kalter, 1986).

Other attempts have also been made to simplify and modify the traditional immunoassay. U.S. Patent No. 5,695,928 disclosed an immunoassay capable of the rapid detection of a variety of test substances in a test sample. The feature of the invention is that extraction and isolation of the test substance occur simultaneously with the formation of the primary antigen-test substance complex. The primary antigen-test substance complex is then captured in a solid phase format having a plurality of interstitial spaces which facilitate rapid and efficient detection.

U.S. Patent No. 5,630,924 disclosed compositions, methods, and apparatus for performing ultrafast binding assays by capillary electrophoresis or other electroseparation techniques.

U.S. Patent No. 5,565,365 disclosed a system for assaying a fluid sample by detection of radiation emitted from a ligand/conjugate complex formed on a plurality of beads dimensioned within a specified range of diameters. The beads are disposed as a porous mass in conduit adjacent to a fluid-porous screen having pores of lesser diameter than the range of diameters of the beads. A plurality of paramagnetic particles is suspended across the conduit by a magnetic field of sufficient intensity to array the paramagnetic particles as the fluid-porous screen.

U.S. Patent No. 5,554,340 disclosed an assay system for a fluid sample, typically employing a fluorescent tag. The system comprises a lens capable of focussing both excitation and fluorescent radiation, a fluid-flow conducting conduit being provided in the lens extending transversely of the optical axis of and through the focal region of the latter. One or more mechanical screens are disposed adjacent to the focal region in the conduit to arrest passage of beads as a function of bead diameter. The beads, precoated with at least a moiety of a ligand/conjugate complex, e.g. a specific-binding ligand, are preferably substantially transparent to both the excitation and fluorescent radiation.

U.S. Patent No. 5,212,065 disclosed a rapid immunoassay device comprising a single porous membrane that serves as both a reagent support and a spent reagent reservoir. The immunoassay device directs the flow of sample and reagents within the device in a manner that eliminates both lateral diffusion and backflow of reagents without the necessity of additional external means.

U.S. Patent No. 5,236,824 disclosed an *in-situ* laser magnetic immunoassay (LMIA) method which eliminated the step of B/F separation generally required in the labeling method of immunoassays. The laser magnetic immunoassay permits a quantitative determination of a target immunological substance, for example, antigens, antibodies, lymphocytes, viruses, tumor cells and infected cells, in an analyte solution containing both bound and free species. A transitory increase in the magnetophoretic scattering of a laser beam is observed when the analyte solution contains magnetic-labeled, bound target analyte, while no such increase is observed in a control test solution containing only the relevant reagents. A magnetophoretic LMIA apparatus is provided which includes a magnetic gradient-generating device, which forms an integral part of the *in-situ* LMIA.

An alternative method to perform the assay of antigen or antibody is the use of an antigen/antibody-based biosensor. Biosensors offer the advantages of inexpensive equipment that

can be taken out of the laboratory to where the sample may be added by unskilled personnel; the result would ideally be available rapidly. Immunosensors can detect the antigen/antibody concentration either by direct competitive and displacement reactions similar to the immunoassays or by direct changes in transducer output (Karube and Suzuki, 1986). For the former type of system, the range of sensor transduction includes optical, amperometric, and radiochemical mechanisms. Similar to the traditional immunoassay, these detection methods generally require the use of labeled receptors and several preparative steps to the overall assay. An alternative sensor system, which can monitor the antigen-antibody interaction by direct changes in transducer output, is a mass-detection sensor. This sensor system can monitor an antigen/antibody reaction directly by the detection of mass change. The assay concept and procedure are simple and any use of potentially hazardous materials is eliminated. An example for this type of system is the piezoelectric (Pz) crystal device. With this system, assays both in gas phase and in solution are possible.

A Pz crystal device consists of a quartz crystal wafer sandwiched between two metal electrodes. The electrodes provide means of connecting the device to an external oscillator circuit that drives the quartz crystal at its resonant frequency. This frequency is dependent on the mass of the crystal, as well as the mass of any layers confined to the electrode areas of the crystal. Changes in mass on the surface of the electrode thus change the frequency of the Quartz Crystal Microbalance (QCM) device. The use of a piezoelectric (Pz) oscillator as a potential biomedical sensor is based on the relationship of the frequency change and the mass loading on the surface of the crystal described by the following equation:

$$\Delta F = -2 F_0^2 \Delta m / A (\rho_q \mu_q)^{1/2}$$

where ΔF is the measured frequency shift, F_0 is the fundamental frequency of the Pz crystal, A is the area coated, Δm is the mass change due to surface deposition, ρ_q is the density of the quartz crystal (2.648 g cm^{-3}) and μ_q is the shear modulus (2.947×10^{11} g cm s^{-1} s^{-2} for AT-cut quartz crystals). The mass sensitivity can be described as:

$$S = 2 F_0^2 / (\rho_q \mu_q)^{1/2}$$

For a 10 MHz piezoelectric crystal, mass sensitivity S = 0.227 Hz ng^{-1} cm^2, predicts that 4.4 ng/ cm^2 of adsorption leads to a frequency change of about 1 Hz.

Pz crystal based immunosensor technology is the combination of the use of a Pz device, protein immobilization, and antigen-antibody interaction. The key to constructing a Pz

immunosensor is the surface modification by which a sensitive antigen or antibody receptor layer is created so that the target analyte from the sample can be adsorbed selectively.

Methods of protein immobilization used in the fabrication of a biosensor include physical adsorption onto a support (metal or polymer), entrapment within a membrane, and covalent binding to the support (Williams and Blanch, 1994). All methods have distinct advantages, for example, a physical method is experimentally simple and regarded as a mild coupling method that preserves protein activity. However, in certain situations, it can be somewhat reversible and does not provide as high a surface loading of protein as covalent coupling. In covalent immobilization methods, chemical bonds are made between the surface and the attached species. Although generally harsher than other immobilization schemes, covalent binding provides the highest irreversible surface loading which is beneficial for the sensitivities of the sensor. Moreover, covalently bound proteins are relatively resistant to the operational conditions. In general, the preliminary requirements for the protein immobilization are the maintenance of activity, a sufficient amount of binding, and strong adhesion of the coated protein on the coated support. Different immobilization methods provide different sensor performance and are suitable for different types of proteins to be immobilized.

The first use of an antigen as a Pz crystal coating was demonstrated by Shons et al. (1972) and was based on adsorption of antigen on a low energy plastic coating, nyerbar C (30%) solution in 1-3 di[trifluoromethyl] benzene, providing a layer capable of forming hydrophobic bonds with proteins. After coating with antigen, the crystal was exposed to an unknown amount of antibody solution. The immunoreaction between antibody and antigen-coated crystal resulted in the immunobinding of the antibody. Frequency shift caused by mass loading is proportional to the concentration of the specific antibody in solution.

U.S. Patent No. 4,242,096 disclosed an indirect immunoassay for detecting an antigen in a liquid sample, in which an antigen was covalently coated onto a crystal having a polymer monolayer such as poly(2-hydroxy-3-dimethylamino-1,4-butane). The using of a polymer priming surface followed by adsorption of the antigen was also disclosed in U.S. Patent Nos. 4,236,893 and 4,314,821, among others. U.S. Patent No. 4,735,906 disclosed a method for protein immobilization using a crystal with a surface modified to form a layer of siloxane polymer, which in turn immobilizes protein through amide linkages. Bastiaans (U.S. Patent No. 4,735,906) disclosed a method for an immunoassay using ST-cut piezoelectric SAW devices in

a solution phase. The surface of the crystal is modified by silane derivative, glycidoxypropyltrimethoxysilane (GOPS). Singapore Patent Application No. 9801211-5 (S. F. Y. Li) disclosed the self-assembly immobilization technique by which the performance of a sensor system was greatly improved compared with other immobilization methods.

Recombinant technology plays an important role in protein biotechnology, by which large quantities of recombinant proteins can be generated as the products of genetic engineering. The aim of producing recombinant proteins can be grouped into four broad categories (Franks, 1993): 1) To obtain large quantities of a protein; 2) To study site-directed mutant proteins; 3) To produce proteins for biotechnology; 4) To manipulate metabolism *in vivo*. In particular, for example, the development of an enzyme immunoassay requires large quantities of structurally well-defined proteins as immunogen and enzymatic tracer. In recent years, there has been growing interest in the use of viral or bacterial specific antigens, produced by recombinant techniques, as protective immunogens in serological tests. For purposes of this disclosure, a recombinant protein or recombinant antigen is a protein or antigen prepared from an organism or cell or from a descendant of an organism or cell into which a gene for the protein or antigen has been cloned and wherein the recombinant protein can be a fusion protein.

The present invention combines the Pz transducer together with the recombinant protein based immunoassay to perform diagnoses for viral or bacterial disease in animals. The fabricated Pz crystal sensor can thus monitor antigen-antibody interaction directly by sensing the mass change of the crystal. It has been proved to be helpful in overcoming the limitations of traditional immunoassay methods. The obvious advantages provided by the novel technology are the following: 1) The system has a fundamental low cost and can be performed in a non-equipped lab; 2) The fundamental design and performance are relatively simple; 3) The ease to realize real-time display of the results and rapid on-site testing; and 4) Minimal usage of hazardous material and use of unlabeled reagents which, unlike a conventional labeled immunoassay, is less temperature sensitive and allows for stable storage and shipment at 20-25°C.

Salmonella enteritidis (SE) is a major problem for the poultry industry. In the last ten years SE has been one of the dominant *Salmonella* serotypes isolated from humans with food poisoning in many countries. This phenomenon has been associated with an increase in the numbers of poultry and eggs contaminated with these serotypes. Bacteriological techniques for the isolation of *Salmonella* from clinical and environmental samples are laborious, lengthy, and

expensive. They may not identify all *S. enteritidis*-infected flocks because of the intermittent nature of *Salmonella* extraction and the number of samples that can be processed. However, chickens infected with invasive serotypes like *S. enteritidis* develop a persistent immunoglobulin G response to the infecting organism. Thus, mass serological screening for a flock infected with *S. enteritidis* offers a relatively cheaper and more practical alternative, provided that it gives at least the same performance as bacterial methods. Thorns et al. (1996) established ELISA-based serological tests for *S. enteritidis*. This method is based on the traditional solid phase immunoassay in which a novel fimbrial antigen termed SEF 14 was first described in strains of *S. enteritidis* and pre-coated on the solid phase. Under the typical ELISA procedures SEF14 specific antibodies from chicken sera and egg yolk were detected qualitatively. U.S. Patent No. 4,689,295 disclosed a method of detecting the presence of *Salmonella* in food samples. It includes providing at least one DNA probe which is capable of selectively hybridizing to *Salmonella* DNA to form detectable complexes, contacting the DNA probes with the bacteria in the food sample under conditions which allow the probe to hybridize to Salmonella DNA present in the food sample to form hybrid DNA complexes, and detecting the hybrid DNA complexes as an indication of the presence of Salmonella in the food sample.

Porcine reproductive and respiratory syndrome (PRRS) is an economically important disease in pigs worldwide. The PRRS virus (PRRSV), a positive-stranded RNA virus belonging to the genus Arterivirus, causes this disease. Current diagnosis for the disease is based on: 1) clinical signs, which are used as characteristic in acute outbreaks, but are not as effective in diagnosing low-grade disease; 2) virus isolation, which is little used as it is time consuming and less reliable; 3) polymerase chain reaction (PCR) testing for PRRS antigen; and 4) serological tests used for herd profiling, which include immunoperoxidase monolayer assay (IPMA), indirect enzyme-linked immunosorbent assay (ELISA), and indirect immunofluorescence assay (IIFA).

IDEXX Laboratories, Inc. of Maine, U.S.A., has a series of antibody diagnostic tests for the detection of alternative virus or bacteria infectious diseases of animal. *Salmonella enteritidis* antibody test kits and PRRS antibody test kits from IDEXX rely on competitive and sandwich enzyme linked immunoassay (ELISA) techniques, respectively. In these specific antibody test kits, antigen-coated plates and all reagents (including enzyme-conjugated antibody, substrate, sample diluent, washing buffer, positive/negative control, etc.) are provided. The test protocol comprises procedures of sample incubation, enzyme conjugated antibody incubation, substrate

incubation, and multi-washing steps. The final signals are measured by spectrophotometer. The whole procedure (from contacting the detected sample to the coated plate) takes 3-5 hours, costs US$3-5 each test, and a well-equipped laboratory and skilled personnel are needed to perform the assays. Although these assays are proved to be highly accurate, sensitive and specific, the above mentioned limitations make it difficult to perform a large number of on-site screening tests under field conditions.

OBJECTIVE OF THE INVENTION

It is therefore an object of the present invention to provide an apparatus and method for diagnosis of veterinary diseases, which involves the detection of viral or bacterial antigen specific antibodies. It is also an object of the invention to detect viral or bacterial specific antigens.

It is therefore an objective of the present invention to provide a method to fabricate a Pz crystal based sensor by using recombinant protein as receptor material and to perform detection for antigen specific antibodies.

It is another objective of the invention to provide a simplified and shortened method for the detection of *Salmonella enteritidis* and PRRSV infection disease.

SUMMARY OF THE INVENTION

The present invention provides a method for an immunodiagnostic test for veterinary diseases, which involves the detection of the presence of viral or bacterial antigens or antibodies to them. The apparatus according to one aspect includes an immunosensor which uses a Pz crystal as reaction carrier. According to another aspect, the recombinant viral or bacterial antigen is immobilized on the surface of the crystal to work as a sensing receptor. A Pz crystal device can sense a mass change caused by the antigen-antibody interaction on the surface of the crystal.

The *Salmonella enteritidis* (SE) or PRRSV detection method includes fabricating a Pz sensor by covalently or physically immobilizing a recombinant SE or PRRSV protein on the surface of the crystal. The technique adopted is either dip- or drop-coating by which the use of minimal amount of the protein is taken into consideration. After blocking by certain reagents the coated interface is exposed to the specimens to be detected (e.g., chicken serum, egg yolk, pig serum), which are suspected to contain SE or PRRSV antibodies for an appropriate period of

time (from several seconds to several hours). The frequencies of the fabricated crystal before and after incubation with a specimen are measured. The frequency change or absence of a frequency change indicates the presence or absence of the target antibodies. Besides the protein coupling procedure, other technique details including washing buffer, blocking reagent, and the dilution ratio of the detected specimen are disclosed.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 is a schematic of the Pz crystal sensor system

Figure 2 is a flow diagram showing the procedures for constructing a Pz sensor with a specific sensing material layer on it and procedures for running the samples.

Figure 3 is a diagram of a microcontainer used in the dip coating process.

Figure 4 shows the principle of a Pz sensor for the detection of a target antibody.

Figure 5 shows the frequency change caused by positive and negative controls for detecting *Salmonella enteritidis* antibodies.

Figure 6 shows the frequency change for 35 unknown chicken sera samples tested for *Salmonella enteritidis* antibodies.

Figure 7 shows SE-Pz sensor detection results for 7 previously characterized negative egg yolks and 12 previously characterized positive egg yolks.

Figure 8 shows the relationship between protein binding amount and sensitivity of the fabricated Pz sensor.

Figure 9 is a summary of measurement results for 41 pig sera. Group A is real negative samples, group B is positive controls and group C is unknown samples.

Figure 10 shows the reusability of fabricated PRRSV Pz sensors.

Figure 11A shows the sensitivity of a regenerated PRRSV Pz sensor. Figure 11B shows the sensitivity of a regenerated SE Pz sensor. In both figures ○ is positive control 1 and ● is positive control 2.

Figure 12 shows the regeneration ability of dichromic acid solution (●) and hot Piranha (○) with a used SE Pz sensor with a thiol compound treated surface.

Figure 13 shows the regeneration ability of dichromic acid solution to thiol compound ( ) and γ-APTES (○) treated crystals.

DETAILED DESCRIPTION OF THE INVENTION

The detailed procedures of a Pz crystal device as a sensor system can be understood by reference to the drawings.

The conventional Pz crystal device design (see Figure 1) has a metal electrode **20** deposited on each of the two sides of the wafer **22**. These two electrodes are connected to an oscillation circuit **24** and a frequency counter **26** for generating and detecting resonant frequencies, respectively. Standard methods such as that described by Shons et al. (1972), use a thin disk of AT-cut quartz crystal containing two electrodes on the double sides. Due to the piezoelectric properties and crystalline orientation of the quartz, the application of a voltage between these electrodes results in a shear deformation of the crystal. These electrodes are used to induce an oscillating electric field perpendicular to the surface of the quartz wafer. The oscillating field produces a mechanical oscillation, a standing wave, in the bulk of a quartz wafer. A resonance oscillation is achieved by including the crystal in an oscillation circuit, where the electric and mechanical oscillations are closed to the fundamental frequency of the crystal. The oscillation circuit and the frequency counter (such as HP 5213A from Hewlett Packard, USA) are well known in the art, and are described in detailed by Breckenstein and Shay (1985). The fundamental frequency depends on the thickness of the wafer, its chemical structure, its shape, and its mass, and can be determined by using a universal counter. The most commonly used crystals are 5, 9, or 10 MHz quartz in the form of 10-16 mm disks that are approximately 0.15 mm thick. Metals are often gold, silver, aluminum, or nickel. In the present invention, the crystal is an AT-cut 10 MHz quartz wafer of 14 mm diameter and 0.2 mm thickness. The gold electrode is 5.1 mm diameter and 100 nm thickness. This crystal offers a mass sensitivity of about 0.902 ng/Hz.

To construct a Pz crystal device as an immunosensor system, the procedures include fabrication of the sensor and the detection of the samples. The fabrication of a Pz crystal as a sensor usually involves surface modification by which a specific bioreceptor interface is created. The assay is based on the transformation of a specific interaction into a frequency signal. When exposing the fabricated sensor to samples, the interaction between a coated interface and a detected analyte results in a change of frequency signal, which can be used to quantitatively or qualitatively identify the target analyte directly.

Figure 2 shows the detailed procedures for both constructing a Pz crystal based biosensor and the testing of sample.

First, the crystal, more particularly the surface of the metal electrode, must be cleaned by washing with an appropriate acid, base, and organic solution. To meet with different surface modification procedures, the appropriate washing methods are adopted. For example, to build a thiol compound based self-assembly monolayer, hot Piranha solution is usually the first method to be considered. Hot Piranha solution can get rid of any oxidizing agent, and as a result a hydrophilic gold surface can be obtained. An alternate washing procedure, which comprises soaking the surface of the crystal in base and acid alternately, can be used for polymer modification. After soaking in base and acid, rinsing steps with distilled water and organic chemicals are performed. The fundamental frequency of the cleaned crystal can be measured as F_0 under dry condition and atmospheric pressure.

In the present invention, several conventional protein immobilization methods are adopted including direct physical adsorption onto the bare metal electrodes, covalent binding on the silane treated crystal, covalent binding on a thiol compound modified surface, and physical adsorption on a hydrophobic polymer (polystyrene) modified crystal.

The physical adsorption of protein on the bare gold surface is based on the strong and irreversible hydrophobic and thiol-gold interaction (Horisberger and Vauthey, 1984). This method is experimentally simple and it does not require any chemical step to modify the gold surface. The adsorption is achieved by incubation of a hot Piranha solution treated crystal (F_0) directly with the solution containing biomolecules and allows the molecules to be adsorbed to the surface of the crystal over an appropriate period of time. The resonant frequency after incubation with the biomolecule is determined as F_B. F_0-F_B is related to the amount of biomolecule binding.

Covalent immobilization is also commonly employed. The procedures of covalent binding normally include modification of the crystal by an appropriate first chemical by which a desired functional group can be introduced onto the surface of the crystal, and covalent bonds can be made between the surface and the attached species. The most commonly used activating functional groups include primary amines, thiols, hydroxyls, and carboxylic acids. For example, polyethylenimine (PEI) or γ-aminopropyltriethoxysilane (γ-APTES) modification provides the crystal surface with an amino group; any thiol compound with a terminal -COOH group can

provide the crystal with a -COOH modified surface. To activate the functional chemical modified surface, a second chemical is sometimes employed as a cross-linking or coupling reagent. Normally the cross-linking or coupling reagents are used to provide additional chemical linkages between the active biomaterial with the modified surface or to activate the modified surface further.

In a traditional immunoassay technique, the most widely used solid phase is the 96-well microtiter plate manufactured from a hydrophobic polymer such as polystyrene (inflexible "rigid" plates) or polyvinyl chloride (PVC, flexible plates). The antigens or antibodies can be attached to the plastic surfaces easily by the hydrophobic interaction between nonpolar protein substructures and the polymer (Crowther, 1995). In Pz sensor fabrication, the binding of protein onto a hydrophobic polymer modified crystal is based on the use of the idea in this reference. This immobilization concept is quite similar with the traditional immunoassay and is easily accepted and understood by those who have a background in immunology. Compared with covalent immobilization, this method is relatively simple since only one polymer modification procedure, use of the first chemical, is needed before the crystal is exposed to the protein to be immobilized.

Although there are a number of methods for biomolecule immobilization, many are deficient for one or more reasons. In general, the method of choice depends on the properties of the biomolecule to be immobilized and the desired performance of the sensor.

To achieve either the chemical modification or the protein immobilization, several coating techniques can be adopted which include dipping, dropping, and spinning. The dip-coating method relies on physical or chemical adsorption of materials to be coated onto the crystal from solution in a suitable solvent of suitable concentration. This procedure is easy to perform just by dipping the crystal into the solution of the chemical/biochemical to be coated for an indicated time. One problem encountered with this technique is the requirement for relatively large quantities of the solution, so that the whole crystal can be immersed into it. The drop-coating method is based on the application of coating material onto the surface of the crystal as a droplet of a volume of several microliters. The former technique can ensure a desired long period of interaction between the crystal surface and the solution and provide a sufficient contact between the surface and the solution. The latter one, however, has a limitation of interaction time because of the spreading and evaporation of solvent and there can be a problem of non-

uniformity of contact between the crystal surface and the solution drop. But the latter method is suitable for polymer coating since the loading of the polymer can be readily calculated from the solution concentration and droplet volume. An alternative method quite suitable for polymer coating is referred to as spin coating. In this method the polymer, dissolved in a suitable solvent, is dropped onto the crystal while it is being spun at high speed. As a result the solution spreads out to form a thin, uniform film across the surface of the crystal which, on evaporation of the solvent, leaves a polymer film over the surface of the crystal.

During the whole procedure of fabricating a Pz immunosensor, the above mentioned coating techniques are adopted individually or combined depending upon the different coating materials and different reaction conditions required.

Figure 3 is a diagram of a container which is suitable for dip-coating the crystal with a minimal amount of precious protein. It is made of microscope slides adhered by Araldite with specially designed dimensions 54 mm×12 mm×1 mm, which is suitable for dipping three crystals at the same time. By using this special container, 200 μL of the coated protein is enough for each crystal. Under optimal protein concentration, micrograms of extracted protein is enough for coating each crystal.

In using an immunoassay and immunosensor, prior to running the samples care must be taken to prevent the non-specific adsorption of interfering proteins to the coated solid phase or fabricated sensor transducer. The presence of non-specific adsorption may result in undesired adsorption of interfering proteins when processing the sample detection. A commonly used method to prevent non-specific adsorption involves incubating of the coated surface with blocking reagents, by which the non-occupied active residues can be occupied by non-activated protein. In a traditional immunoassay bovine serum albumin, human serum albumin, casein, gelatin, detergents, and nonfat dried milk, etc., are commonly used to practice this aim (Crowther, 1995). In the present invention, appropriate blocking reagents are adopted to meet with requirements for different situations. The blocking is achieved either by dipping the coated crystal in a blocking reagent or dropping a droplet of the said blocking solution onto the coated surface. This procedure is performed after the sensing protein immobilization, so that when incubated with the detected samples any mass attachment not caused by specific immunoreaction can be prevented.

The purpose of washing is to separate bound and unbound (free) reagents. During the sensor fabrication stage, washing steps are achieved with the solvent used in the relevant coating procedure and followed by distilled water. To monitor the whole coating process, F_0, F_1, and F_2 are measured in a dry state. The ΔF between two successive procedures corresponds to the absolute amount of the material attached, which can be calculated on the basis of the Saraubery equation. The uniform frequency change of each immobilization procedure between individual crystals will lead to a uniform amount of protein binding between individual crystals, and furthermore, the ability to reproduce the sample detection can be ensured.

The washing solution used following the biomolecule immobilization and the blocking procedure is usually buffered, typically PBS (0.01-0.1 M, pH 7.4), in order to maintain isotonicity, since most antigen-antibody reactions are optimal under such conditions. Besides the preservation of activity for the immobilized protein, the other critical requirement is the stability of the protein on the sensor transducer, which affects the long term stability and sensitivity of the sensor. In the present invention the multi-washing is needed to ensure the stability of the immobilized protein (F_B', F_B'' ....). The frequency change or lack of frequency change between the multiple washing steps indicates whether the desorption occurred or not.

Figure 4 is a diagram describing the principle of the fabricated Pz sensor with an immobilized sensing protein layer for performing the detection of specific antibodies from animal body fluids. **28** refers to a Pz crystal; **30** is the coated sensing protein (antigen); **32** is the target antibody which is specific to the coated antigen; **34** is the blocking non-active protein; **36** represents other antibodies; and **38** represents other serum components. This detection procedure according to the present invention includes the following steps: 1) obtaining the resonant frequency of the coated and blocked crystal at dried state F_R; 2) incubating the test sample solution with the reactive surface of the crystal; 3) washing away unbound material and drying the crystal; and 4) measuring the resonant frequency of the crystal F_S. The frequency change or lack thereof (before and after incubation with specimen (F_R-F_S)) indicates the presence or absence of the antibodies to antigens, and provides a positive/negative or YES/NO answer for the diagnostic test.

The reaction between antibodies and coated antigens depends on the distribution, time, temperature, and pH at which the incubation step takes place. The temperature for incubation is most commonly 37°C or room temperature. The time of incubation is several minutes to 1 hour.

Incubation steps in the present invention involve either dipping the coated crystal into a specimen or dropping the specimen onto the surface of the crystal and allowing the solvent to evaporate. The time and temperature required depend on the titer of each different immunoreaction system. The buffering condition, in the present invention, is controlled by PBS buffer, which maintains a pH 7.2.

The used crystal can be regenerated and reused several times in either of two ways. If a fabricated crystal is incubated with a negative sample, producing only a small signal change, it can be reused to perform another detection. On the other hand, the used crystals can be regenerated by using certain solutions to strip the coating completely and rebuilding a fresh gold surface. The regenerated crystal can be used to construct any other coating by means of new surface modification. In this sense, the crystals have no limited life.

A Pz crystal based immunosensor has been proved to be inexpensive, simple, and can perform a detection rapidly. Once a sensing layer has been immobilized on the surface of the Pz crystal, the assay time required is several minutes to I hour. Furthermore, this assay requires minimal technical skill, simple procedures, and no sophisticated laboratory equipment.

The present invention is further detailed in the following Examples, which are offered by way of illustration and are not intended to limit the invention in any manner. Standard techniques well known in the art or the techniques specifically described below are utilized.

EXAMPLE 1

The Fabrication of a Pz Sensor for the Detection of *Salmonella enteritidis* Disease in Chickens

A) Preparation of Recombinant *Salmonella enteritidis* (SE) Antigen

Unique sequences encoding for *Salmonella* serotype *enteritidis* in chicken were identified. These allow for the specific detection of *S. enteritidis*. Nucleotides 754-1023 of a clone of a partial gene sequence of a flagellin (GenBank Accession No. Z15068) were utilized giving a 270 bp sequence (SEQ ID NO:1) encoding 90 amino acid residues (SEQ ID NO:2). The 90 aa peptide reacts specifically with sera from chickens infected with *S. enteritidis*. Chromosomal DNA from *Salmonella enteritidis* strain 13076 was used to amplify the sequence by means of polymerase chain reaction (PCR) techniques. The amplified PCR product was inserted into a bacterial expression vector next to a gene for glutathione S-transferase (GST) such

that a recombinant fusion protein (GST joined to the 90 aa peptide) was produced when the gene was expressed. This vector was cloned into bacterial host *Escherichia coli* JM 105 competent cells. A single colony of *E. coli* cells containing the appropriate recombinant plasmid was used to inoculate 1 mL of Luria-Bertani medium with 70 μg/mL of ampicillin (included for the selection of the transformed cells). After 4 hours, 200 μL of this culture was diluted 1:10 and incubated at 37°C with shaking at 250 rpm for 10 hours. This culture was then further diluted 1:10 in an appropriate flask and grown under the same conditions as previously described. When the O.D. 600 of the culture reached 0.6, IPTG (isopropyl-β-D-thiogalactopyranoside) was added to a final concentration of 2.5 mM and incubation was continued for another 4 hours. The bacterial cells were then pelleted and the cell pellet resuspended in lysis buffer (50 mM Tris pH 8, 0.3 mM NaCl, 0.5 mM EDTA, 0.5% Tween 20). The bacterial cells were then sonicated and centrifuged at 10000xg for 20 minutes. The protein was sequentially extracted from the pellet in the supernatant and in 1 M , 6 M, and 8 M urea all dissolved in lysis buffer. Protein at each step of this purification was analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) and visualized by staining with Coomassie brilliant blue. Most of the recombinant protein was recovered in the supernatant and 1 M urea extraction. The recombinant protein in these fractions was then purified through an affinity column for GST using a kit available from Pharmacia Biotech. Further analysis by SDS-PAGE showed 98-99% purity of the protein. The molecular weight calculated from sequence is 9,000 and 35,000 determined by SDS gel electrophoresis. The purified and recombinantly expressed protein was characterized by Western blot with the use of yolk (extracted from chicken eggs) and negative and positive sera from naturally and experimentally infected chickens.

B) Fabrication of the SE-Pz Sensor

In the present invention three conventional protein immobilization procedures were adopted to fabricate the Pz sensor with recombinant *Salmonella enteritidis* protein layer on the surface of the crystal. It was proved that all these methods ensure the same amount of protein binding without loss of activity.

C) Immobilization Via a Self-assembled Monolayer Technique

10 MHz AT-cut mounted crystals with gold electrodes (from ICM Co. Inc., Oklahoma, USA) were cleaned with hot Piranha solution (30% H_2O_2 : H_2SO_4 (1:3)). The Piranha solution was spread on the gold surface for 5 minutes, after which the electrode surface was thoroughly rinsed with distilled water. This was followed by rinsing with 95% ethanol and then with distilled water again. This procedure was repeated twice. The surface was then finally blown dry with a stream of nitrogen gas and the resonance frequency (F_0) was taken.

For covalent binding of said SE antigen, in this experiment, the surface of a gold electrode was first modified by 4-aminothiophenol (ATPh) which provides a thiol group on one side and an amino group on the other side of the molecule. The spontaneous adsorption of thiol group to the gold surface ensures that the amino group faces towards the interface and works as a functional residue to which another amino group from a protein can be immobilized by means of a cross-linking reagent. This said immobilization was achieved by dipping the freshly cleaned crystal immediately into 20 mM of ATPh (the first chemical) in dimethylsulfoxide (DMSO) overnight at room temperature. After washing with distilled water and air drying, the obtained amino groups were further activated by dipping the modified crystal in 2.5% glutaraldehyde (GA) in PBS (the second chemical) for 1 hour. After washing with PBS buffer and rinsing with distilled water, incubation with protein was performed.

Application of a droplet of protein onto the coated crystal is the most commonly used technique to fabricate a sensing layer, particularly in antibody coating. In this experiment, this technique was first investigated. This involved applying 5 µL each of concentrated SE protein solution (500 µg/mL) on each side of the activated crystal and incubating at 37°C until the solvent evaporated completely. Unfortunately, however, this method failed to ensure that all the coated crystal would have the desired amount of protein to be immobilized (corresponding to a certain frequency change). The reasons may be the limited incubation time this method offered and the content of 0.1% SDS in the protein solution which is used in purifying the recombinant protein. To overcome these problems, an alternative dipping coating technique was developed by which the modified crystals were dipped into a diluted SE PBS solution (25 µg/mL) and incubated for a desired period of time (1-2 hours) at 37°C. This technique is based on the design and use of a homemade microcontainer, which is of the size of 54 mm x 12 mm x 1 mm, capacity

of 648 microliters. This special design allows three pieces of crystal to be dipped simultaneously, and typically, 5 μg of recombinant protein is enough for each crystal.

This method is found to be more suitable for recombinant protein coating, for it not only ensures a sufficiently long period of incubation time but also reduces the amount of protein needed. Moreover a longer incubation time allows the use of lower concentrations of coated protein. In this example, use of an SE concentration up to 25 μg/mL, the binding amounts correspond to saturation of the surface, i.e., the frequency stops changing. Furthermore, diluted protein results in a minimal amount of the anionic detergent SDS and prevention of the protein immobilization is reduced. Besides, this dipping method is beneficial in reducing the variation of the amount of protein binding to individually coated crystals since it ensures a sufficient and uniform contact between crystal and the protein solution. The binding amount of SE protein by drop- and dip-coating technique are compared in Table 1.1.

Table 1.1

SE binding amount corresponding to frequency change*

Method	Dropping	Dipping
ΔF (Hz)	165.3±33.2	256±23.3
Binding capacity (nmol/side)	0.0021	0.0032

* Average from at least 8 crystals

D) Immobilization Via Silanization Method

Another type of coating procedure is based on the silanization method. The crystals were cleaned in 1.2 N NaOH for 20 minutes followed by 5 minutes in 1.2 N HCl. The crystals were then rinsed with distilled water and air-dried. Subsequently several microliters of concentrated HCl were applied on the surface for 1-2 minutes. Finally, the crystals were rinsed with distilled water and ethanol.

The clean crystals were first modified by dipping in 5% γ-aminopropyltriethoxysilane (γ-ATPES) in acetone for 1 hour. After washing with acetone and distilled water (F_1), the following procedures are the same as for the APTh method, which consists of further activation by GA (F_2) and finally incubation with 25 μg/mL of recombinant SE protein by dipping (F_B). Multiple washing steps were followed to ensure the stability of the coating (F_B', F_B'').

E) Immobilization Via Polystyrene Method

The crystals were cleaned by the alternating base and acid method, as described previously. Polystyrene beads were dissolved in toluene into which cleaned crystals were dipped for 30 minutes at room temperature to form a polymer film by physical adsorption. After rinsing with ethanol and distilled water (F_1) the crystals with polymer film were dipped into recombinant SE protein in coating buffer for an appropriate period of time (F_B).

The thickness of polystyrene film depends on the concentration of the polymer solution. In this case 3 mg/mL was adopted, and the amount of polymer coating corresponding to the frequency change was 1196±125 Hz. Although 9 mg/mL of polymer provided a thicker polymer coating corresponding to a frequency change of 4919±325 Hz, the amount of protein coating no longer increased. This indicated that 3 mg/mL of polymer provided a saturated polymer coating and the coating covered the whole area of the crystal.

The adsorption of protein onto plastic is based on hydrophobic interactions. The rate of the interaction depends on the concentration of the protein, coating pH, temperature, and time of incubation. In this case the optimal coating procedure was dipping the crystal in SE solution (25 μg/mL in 50 mM carbonate coating buffer, pH 9.6) at 37°C for 1 hour. It is indicated that the concentration of SE up to 25 μg/mL resulted in the saturation amount of binding corresponding to a frequency change of 240±19.9 (Table 1.2).

Table 1.2

Frequency change caused by binding of SE protein under different concentrations

Conc. (μg/mL)	30.0	25.0	12.5	10.0	6.0
ΔF (Hz)	232	240	171	140	55

During the whole coating process, F_0-F_B were taken in order to monitor the reaction procedures. It is proved that the three immobilization methods ensure almost the same amount of SE binding (Table 1.3) without loss in activity. These coating methods were stable, as there was no frequency change during the washing procedures. Under the optimal coating condition, 5 μg of the said protein are enough for the fabrication of one Pz crystal.

Table 1.3

Procedures of SE binding by different methods

	Method		
	ATPh	γ-APTES	Polystyrene
Steps		ΔF (Hz)	
First Chemical	1063±12.8	545±58.6	1196±125
Second Chemical	171±19.1	273±221	NIL
SE protein	256±18.3	232±22.3	240±23.2

In the present invention, a polystyrene-coating method was first recommended as it offers a relatively simple concept and reduction of immobilization time. By means of the said coating process a large quantity of Pz crystals with SE protein coating layer were fabricated to conduct the test for SE specific antibody from chicken serum samples or egg yolk.

To avoid non-specific adsorption, the SE protein coated crystals were incubated with BSA by dropping 5 μL of 5% BSA on each side of the crystal or dipping the crystals in 1% BSA solution at 37°C for half an hour. After washing and drying the said crystals were kept at 4°C or refrigerated.

For SE detection the polystyrene method was selected as the best method and in the following sections (F) and (G) of this Example the Pz crystals used were prepared with the polystyrene coating method. SE protein fixing on a polystyrene modified surface is based on a physical adsorption. It was found that a polystyrene modified electrode surface results in a low activity for binding interfering proteins and consequently nonspecific binding is greatly reduced. The binding of SE to the polystyrene film is due to electostatic interaction which is achieved by a long (overnight) incubation. A short incubation (5-10 minutes) with the serum sample allows only specific antibody binding to the coated antigen.

F) Detection of SE Antibodies from Chicken Sera

The fabricated crystals were taken from the freezer and allowed to equilibrate to room temperature. The resonant frequency was measured as F_R. Sample used in this detection was chicken sera. Diluted serum samples reduced the background caused by interfering protein, but also caused a loss of sensitivity due to the lower antibody concentration. In this example, dilution of serum samples fifty-fold (1:50) with PBS buffer was found to be the best compromise between

the requirements of sensitivity on the one hand and handling of interfering binding on the other hand. Under the optimal dilution, 10 μL of diluted serum sample was placed onto each of the two sides of the crystal and covered the whole area of the electrode at room temperature for about 20 minutes. After washing and drying of the said crystal, resonant frequency values were determined as F_S. F_R-F_S corresponds to the mass adsorption caused by detected serum sample.

A total of 47 chicken sera were involved. Six control negative and 6 control positive sera were used to identify the performance of the fabricated sensor. The results are shown in Figure 5. The frequency change caused by positive and negative samples is obviously different and provides a YES or NO diagnostic result. The cut-off value was set as the average signal of the six negative controls plus three times SD which gave a cutoff threshold of 176.7 Hz in this case.

Another 35 unknown sera were assayed using an SE-Pz sensor (Figure 6) and the results were compared to traditional immunoassay results. For an SE-Pz sensor, the presence or absence of antibody to SE is determined by a frequency change of the crystal before and after incubation with serum specimen. If the ΔF (F_R-F_S) is less than the cutoff threshold, 176.7 Hz in this case, the sample is classified as negative for SE antibodies, and if the ΔF is greater than or equal to the cutoff threshold, the sample is classified as positive for SE antibodies. The positive control serum (serum 0) produced a highly positive signal. Six samples out of 35 were stronger than or as strong as the positive control serum. Another 11 samples appeared as positives and the remaining 18 were negative. These results all agree, except from serum 33, with a Western blot assay and the commercially available IDEXX SE Antibody Test Kit. Table 1.4 is a 2×2

Table 1.4

Summary and comparison of SE-Pz sensor and traditional immunoassay

	Western blot/IDEXX ELISA confirmed		
	Positive	Negative	Total
SE-Pz sensor positive	16	1	17
SE-Pz sensor negative	0	18	18
Total	16	19	
SE-Pz sensor positive	a	b	a+b
SE-Pz sensor negative	c	d	c+d
Total	a+c	b+d	

Sensitivity = a/(a+c); Specificity = d/(b+d); Concordance = (a+d)/(a+b+c+d)

contingency Table, by which the comparison of SE-Pz sensor with the two traditional immunoassay are made. The Pz sensor and traditional immunoassay result concordance was 96%, and the relative sensitivity and specificity were 100% and 95%, respectively.

G) Detection of SE antibodies from chicken yolk

In this example, the detected sample type is egg yolk. Dilutions from 1:1 to 1:10 were tested. It was found that a 1:5 dilution with PBS buffer was optimal for the requirements of sensitivity and handling of the interfering binding. Under the optimal dilution, 10 μL of diluted egg yolk sample were added onto both sides of the crystal and covered the whole area of the electrode in a 37°C incubator for about 30 minutes. After washing and drying the said crystal, a resonant frequency value was determined as F_S. F_R-F_S corresponds to the mass adsorption caused by detected samples. A total of 19 known egg yolks from experimentally infected or uninfected chickens were detected by an SE-Pz sensor. The results are shown in Figure 7. The cut-off value was defined as the average of the negative samples plus three times the standard deviation.

EXAMPLE 2

Fabrication of a Pz sensor for the Detection of Porcine Reproductive and Respiratory Syndrome Virus Disease in Pigs

A) Preparation of Recombinant Porcine Reproductive and Respiratory Syndrome Virus (PRRSV) Antigen

Unique sequences encoding for Porcine Reproductive and Respiratory Syndrome Virus (PRRSV) in pigs were identified. PRRSV has eight Open Reading Frames (ORFs) in its genome: 1a, 1b, 2, 3, 4, 5, 6, and 7. A PRRSV strain was used for amplification of viral ORFs 5 and 7 by PCR and cloned into bacterial host *Escherichia coli* JM 105 competent cells. The ORF 5 DNA sequence is shown as SEQ ID NO:3 and the ORF 5 protein sequence is given as SEQ ID NO:4. The subsequent recombinant expression process and purification procedure were nearly identical to that for the SE antigen described in Example 1. Viral protein products from ORFs 5 and 7 were recovered in the 8 M urea extraction step. The 8 M urea fraction was purified by excision of the band from a preparative SDS-PAGE. The purified and recombinantly expressed protein were 98-99% pure and were characterized by a Western blot with the use of positive and negative

sera from naturally and experimentally infected pigs. The molecular weight calculated from sequence is 9,000, and 41,000 kD determined by SDS gel electrophoresis.

B) Fabrication of a Pz Sensor with PRRSV ORF 5 Antigen Layer (PRRS-ORF 5-Pz Sensor)

In this example, recombinant PRRSV-ORF-5 protein was used as sensing antigen. To fabricate the Pz sensor with a PRRSV-ORF-5 protein layer, covalent and non-covalent immobilization methods were adopted as described in Example 1. Although each method ensured a sufficient amount of protein binding corresponding to certain frequency changes, further experiments indicated that covalent binding is not suitable to distinguish the positive and negative serum samples. Two problems were encountered. The relatively strong background contributed by the negative control and the weak frequency change when incubated with the positive control. The former problem may indicate that the nonspecific adsorption residue of the coated surface still exists as the result of the interfering proteins, the serum component, adsorbed on it. The latter problem may result from loss of activity of the coated PRRSV protein and as a result the immuno-reactions between detected antibody and the coated PRRSV protein were prevented. To overcome these two problems and obtain a clear distinction between positive and negative signals, the following efforts were made. First, appropriate blocking reagents, such as PBS, dried milk, gelatin and casein buffer, were used to incubate with PRRSV coated crystal before contacting the coated crystal with serum samples. Second, to increase the frequency change signal caused by the positive control serum, the serum incubation conditions were modified including the time, temperature, and serum dilution. All these efforts were ineffective in improving the performance of the sensor.

Fortunately, the non-covalent binding of PRRSV protein directly onto the surface of the crystal is quite reasonable for fabrication of the Pz sensor with PRRSV layer.

There is a strong and irreversible adsorption of protein on a gold electrode due to the hydrophobic and thiol-gold interaction between protein molecules and gold surface. To immobilize PRRSV protein onto the surface of the crystal, the thoroughly cleaned crystals (F_0) were dipped into PRRSV protein solution overnight at room temperature. To avoid the evaporation of the solution, the homemade micro-container with dipping crystal was kept in a box at high humidity. After washing with distilled water the coated crystals were air-dried (F_B). Casein buffer, which contains 0.5% casein, 0.2% Tween 20 in PBS buffer, was incubated with

the crystal at room temperature for 1 hour. This was followed by washing and drying (F_R). F_0-F_B corresponds to the amount of protein binding, and F_B-F_R relates to the adsorption of the block protein.

The rate and extent of protein coating on a gold surface depend on: 1) the diffusion coefficient of the coated molecule; 2) the ratio of the surface area being coated to the volume of coating solution; 3) the concentration of the substance being adsorbed; 4) the temperature; and 5) the length of time of adsorption. All these factors are linked and the most important is to determine the optimal antigen concentration for coating in each system. To ensure high sensitivity of the fabricated sensor the coating protein needs to saturate available sites on the surface of the crystal on the one hand. On the other hand care must be taken to assess effects of binding proteins at different concentration, since the actual density of binding may affect results. High-density binding of antigen may not allow antibody to bind because of steric inhibition (the antigen molecules being too closely packed).

The concentration range of PRRSV protein used in this Example was 5-50 μg/mL. In this range the protein binding amount corresponded to a frequency change of 140-800 Hz. After blocking the coated crystals (F_R), a real PRRSV-positive pig serum was incubated with them (F_S) and the sensitivity of the sensors with different PRRSV protein binding capacities was monitored (Figure 8). It was observed that PRRSV binding amount at the range of 196.4-312.5 ng/double side, corresponding to a frequency change of 220-350 Hz, was the most sensitive, with the ΔF (F_R-F_S) caused by the positive control being 260-320 Hz.

To obtain 220-350 Hz of PRRSV binding, 25 μg/mL of PRRSV solution was used as the optimal concentration. Under this condition, a large quantity of crystals were coated. The amounts of protein binding to ten crystals are shown in Table 2.1. The highly uniform amount of PRRSV protein binding for each fabricated crystal is the basis of high sensor-to-sensor reproducibility. This physical adsorption method was used for the experiments described below in sections C-F of this Example.

Table 2.1

ORF 5 PRRSV protein binding amount

No.	ΔF caused by PRRSV binding (Hz)	Binding capacity (ng/side)	Binding capacity (nmol/side)
1	250	112.8	0.0028
2	330	148.8	0.0036
3	285	128.5	0.0031
4	325	146.6	0.0036
5	260	117.3	0.0029
6	310	139.8	0.0034
7	360	162.4	0.0040
8	265	119.5	0.0029
9	280	126.3	0.0031
10	300	135.3	0.0033
Average±SD	296.5±35.0	133.7±15.8	0.0033±0.00038

C) Sample Test Protocol

The coated and blocked crystals were kept at 4°C. The coated and blocked crystals were removed from the refrigerator and allowed to reach room temperature. The resonant frequency was measured as F_R. The samples used in this detection were pig serum samples. In this Example, diluting serum samples five-fold (1:5) with PBS buffer was found to be the best compromise between the requirements of sensitivity and handling of interfering binding. Under the optimal dilution, 10 μL of diluted serum sample were added on each side of the crystal and the whole area of the electrode was covered at room temperature for 10 minutes (it was found that a 10 minute sample incubation is optimal). After washing and drying the said crystal, resonant frequency values were determined as F_S. F_R-F_S corresponds to the mass adsorption caused by the detected serum samples.

A total of 41 pig sera were included in this Example. Figure 9 shows the summarized measurement results in a column graph. Of these samples 12 sera (group A) and 14 pig sera (group B) are negative controls and positive controls, respectively, which were employed to determine the performance of the sensors. 12 true PRRSV-negative reference sera (group A) had normally distributed F_R-F_S values ranging from 0 to 60, with a mean value of 30.0±20.9 Hz (Table 2.2). The cutoff threshold was set as the average frequency shift of 12 negative controls plus three times the standard deviation. Another 15 pig sera (group C) were unknown sera and

were used to compare the sensitivity and specificity of the sensor with traditional Western blot and commercially provided ELISA results. The presence or absence of antibody to PRRSV is determined by a frequency change of the crystal before and after incubation with the serum specimen (F_R-F_S). If the ΔF is less than cutoff threshold, the sample is classified as negative for PRRSV antibodies, and if the ΔF is greater than or equal to cutoff threshold, the sample is classified as positive for PRRSV antibodies. 8 samples out of the 15 in group C were positive and the rest were negative. These results all agreed well with the IDEXX PRRS Antibody Test Kit or Western blot assay.

Table 2.2
Distribution of ORF 5 PRRS Pz sensor
frequency change value for real negative pig serum samples

No.	ΔF (Hz)
1	60
2	5
3	52
4	32
5	10
6	40
7	7
8	2
9	36
10	50
11	9
12	55
Average±SD	30.0±20.1
Cutoff threshold (Average + 3 SD)	90.2

D) Cross Comparison between IDEXX ELISA

Several real pig sera results obtained by an ORF 5 PRRSV Pz sensor were cross-compared with the results of the commercially available IDEXX PRRS ELISA TEST KIT (Table 2.3). The IDEXX PRRS ELISA procedures were performed using the standard protocols, by which the presence or absence of antibody to PRRSV is determined by calculating the S/P ratio of each sample. If the S/P ratio is less than 0.4, the sample is classified as negative for PRRSV antibodies, and if the S/P ratio is greater than or equal to 0.4, the sample is classified as positive for PRRSV antibodies.

Table 2.3

Cross comparison between PRRS-ORF 5-Pz sensor and IDEXX ELISA

		PRRSV Pz sensor		IDEXX PRRS ELISA	
		ΔF (Hz)	Normalized signal *	S/P ratio	Normalized Signal*
Cut-off threshold			90.2		0.40
Pig Serum Samples	1(-)	0	0	0.10	0.25
	2(-)	10	0.11	0.17	0.41
	3(-)	35	0.39	0.24	0.60
	4 Weak (+)	155	1.72	0.73	1.83
	5 Weak (+)	140	1.55	0.63	1.58
	6(+)	310	3.44	2.0	5.00
	7(+)	250	2.78	2.1	5.25

The normalized signal of each sample is defined as the ratio of the frequency change of the sample, ΔF_{sample} or $(S/P)_{sample}$, to cutoff threshold frequency. Each serum in Table 2.3 with a measurement normalized value larger than 1 can be classified as a positive specimen. Serum samples 1-3 were identified by a Pz sensor as negative with a normalized signal range from 0-0.39, whereas the normalized signal from the IDEXX ELISA was in the range of 0.25-0.60. Serum samples 6 and 7 were strong positives with a normalized signal from the Pz sensor of 2.8-3.4, and the IDEXX ELISA gave a normalized signal of 5.00-5.25. Serum specimen Nos. 4 and 5 were identified as weakly positive. The normalized signals for both the PRRSV Pz sensor and the commercial IDEXX ELISA were only a little bit higher than 1.

E) Reproducibility of the ORF 5 PRRSV Pz Sensor

To monitor the reproducibility of frequency change from sensor to sensor, several sera samples were detected multiple times with different fabricated sensors (Table 2.4). The standard deviation was in the range of 5.0-15.0%, which was comparable with IDEXX results and were acceptable.

Table 2.4

Parallel detection of serum samples by different Pz crystals

Sample						
ΔF (Hz)	1 (-)	2(-)	3 (Weak +)	4 (+)	5(+)	6(+)
Times						
1	90	10	140	310	225	295
2	55	20	115	250	195	310
3	60	5	--	320	250	--
4	--	--	--	240	--	--
Ave.±SD	67.8±9.9	15.3±2.3	127.5±17.7	275.5±23.3	223.2±27.5	302.5±17.5
RSD (%)	14.6	15.0	13.8	8.5	12.3	5.3

F. Reusability of the sensors

The reusability here refers to the case in which a fabricated crystal was used to detect a negative serum first, and it could be reused to perform another assay. Four fabricated crystals (1-4) were employed to detect two negative control sera, and two positive control sera in different order. The result is shown in Figure 10. Crystal 1 was incubated with negative control 1 first, with the resulting signal, ΔF, being 36 Hz. Crystal 1 was then incubated with negative control 2 and produced a 50 Hz frequency change. Furthermore, this twice-used crystal was then incubated with a positive control and the produced signal was 225 Hz. Crystal 2 was used as a reference, by which the same positive control was detected directly, and produced a 250 Hz signal. Another two crystals were used to perform a similar experiment. Positive control 2 was detected by a once-used crystal and directly by a new crystal. The produced frequency signals from the two tests were very close to each other, and the SD was within the proposed SD, 15%. This indicated that fabricated crystals can be reused at least 3 times without obvious loss of sensitivity.

Example 3

Regeneration of Used Crystals

After a positive sample assay, the regeneration of fabricated crystals can be performed by a pH-shift. Soaking the used crystal for 30 minutes in buffered boric acid/KCl-NaOH, pH 11.0 (50 mL of 0.025 M borax + 22.7 mL 0.1 M NaOH) can remove the antibody from the immunocomplex. After washing with distilled water and PBS buffer the crystal can be used for

another assay. Figures 11A-B show the sensitivity of a regenerated PRRSV-Pz sensor (Figure 11A) and a regenerated SE-Pz sensor (Figure 11B). About 3-4 assays can be performed before irreversible loss of activity occurred.

Dichromate acid (10 g potassium dichromate dissolved in 30 mL hot H_2O, let cool, add 70 mL concentrated H_2SO_4) washing solution was found to be the best way to strip the whole coating from a fabricated sensor surface. It is suitable for almost all surface conditions. For example, a thiol compound modified surface has a strong linkage between gold and a sulfur atom. Treating the said surface by dropping 10 μL of dichromate acid solution for more than 15 minutes, followed by rinsing with distilled water can make the frequency return to the original base line F_0, and any new surface modification is permissible on the newly built gold surface. Figure 12 shows the comparison of regeneration ability of dichromate acid solution and hot Piranha for the thiol compound treated surface. Recombinant PRRSV protein immobilization directly on gold is proved to be rather stable. The regeneration for a PRRSV adsorbed gold surface involves incubation of the surface with dichromic acid for 15 minutes.

The interaction of γ-APTES on gold is based on three gold and oxygen linkages. Regeneration of a γ-APTES treated crystal can be achieved by washing for a long length of time. Figure 13 shows the regeneration ability of dichromate acid washing solution for APTh and γ-APTES treated crystals.

While the invention has been disclosed in this patent application by reference to the details of preferred embodiments of the invention, it is to be understood that the disclosure is intended in an illustrative rather than in a limiting sense, as it is contemplated that modifications will readily occur to those skilled in the art, within the spirit of the invention and the scope of the appended claims.

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